



Saskatchewan
Agriculture
and Food

FINAL REPORT

FIELD TEST METHOD FOR BLUE-GREEN ALGAE TOXINS

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Detection of microcystins in contaminated water supplies using a field kit

Toxic cyanobacterial blooms which are found in natural water supplies are known to produce microcystins. Microcystin-LR, a microcystin analogue, is a potent tumor promoter in the liver. Therefore, contaminated water supplies pose a significant health risk to humans and animals worldwide.

We have developed a prototype field kit for detection of microcystins. The kit is based upon a colourimetric protein phosphatase-1 (PP-1) inhibition assay. In the absence of toxin, PP-1 reacts to give a clear yellow colour in a test tube. In the presence of toxin, this colour reaction is inhibited, giving a colourless solution. The difference in color development with and without microcystin is visible to the naked eye.

Materials

Recombinant PP-1 was expressed in *E. Coli* and purified by standard column chromatography techniques. The pure enzyme was fully characterized for its responsiveness to microcystins by employing a laboratory colourimetric inhibition bioassay. This assay was calibrated to detect microcystins at concentrations between 0.1 and 1.0 $\mu\text{g/L}$ in a 200 μl water sample. A standard curve for inhibition is shown in Figure 1. Microcystin standards were calibrated using an independent procedure involving quantitative amino acid analysis. In our assay, the IC_{50} for microcystin inhibition corresponds to a microcystin concentration of 0.36 $\mu\text{g/L}$, which is equivalent to a potency of 0.29 nM. This value corresponds closely to the IC_{50} for microcystin against recombinant PP-1 reported by other laboratories.

Proposed Field Kit

We have developed a field kit for a single sample analysis. The kit contains:

- A plastic syringe with a millipore filter attachment used for collecting and filtering water samples.
- Distilled water sample (2ml) which is used for the negative control reaction.
- Four clear 8ml graduated test tubes with caps.
 - Tube #1: contains Tris reaction buffer (69 μl , pH 8.3).
 - Tube #2: contains β -mercaptoethanol (4.4 μl).
 - Tube #3: contains PP-1 (67 μl , in 50% glycerol).
 - Tube #4: contains para-nitrophenyl phosphate (pNPP) (267 μl).



Procedure

1. Place 2 ml water into syringe, then place a millipore filter on the end of the syringe. Push water through the filter into tube #1. Mix thoroughly by inverting several times. Make sure to mix thoroughly after each procedure before moving on to the next.
2. Add the contents of tube #1 into tube #2. (Mix well)
3. Add the contents of tube #2 into tube #3. (Mix well)
4. Add the contents of tube #3 to tube #4. Tube #4 is now the reaction tube and is placed inside a 50ml plastic container that is lined with a thermal warming sachet. This warming container provides 50 °C heat for the duration of the reaction which is 30 minutes.
5. In each kit a negative control reaction (distilled water sample) will be set up in parallel using the above procedure.

Results

The kit was applied to the evaluation of distilled and contaminated (1.5µg/L Microcystin-LR) water samples. Figure 2 shows the outcome after leaving the reaction tubes in the heat pack incubating for 1 hour. Tube A which has the distilled water appears as a yellow solution. Tube B with the contaminated water sample shows the colour reaction being inhibited by the toxin, giving a colorless solution.

These tubes were then left at room temperature for several hours to see if there was a more dramatic colour change. The tube that contained distilled water appeared as a bright yellow solution (Figure 3, Tube A), while the 1.5µg/L Microcystin-LR water sample appeared as a pale yellow solution (Figure 3, Tube B).

Practical Considerations

Stabilizing the activity of PP-1 has been a major part of this project. The activity of PP-1 is remarkably preserved in the presence of 50% glycerol.

- A few months ago we found that at -20°C PP-1, diluted and added to tube #3 in the exact concentration that would be used in the kit, is stable for at least 6 weeks.
 - ➔ Now our new findings show that PP-1 diluted is stable for at least 10 weeks at -20°C. In order for the enzyme to remain stable, the following conditions must be attended to:
 - The stock tube must not be opened too many times, because this can lead to evaporation.
 - The stock tube should not be taken in and out of the -20°C freezer too often.
- At +4°C PP-1, diluted and added to tube #3 in the exact concentration that would be used in the kit, is stable for 11-14 days.

- At room temperature PP-1 is stable for 6-10 days.
- Thiol reagents such as β -mercaptoethanol (BME) and Dithiothreitol (DTT) can be used to prevent oxidation of PP-1. DTT and BME have been tested in our laboratory. BME is preferred, although it has a stronger odour, as DTT produces a precipitate in the reaction mixture.
- We are still working on narrowing the 4 step procedure to 3 steps. The kit would then contain:
 - Tube #1: Tris reaction buffer (69 μ l) and β -mercaptoethanol (4.4 μ l).
 - Tube #2: PP-1 (67 μ l, in 50% glycerol).
 - Tube #3: para-nitrophenyl phosphate (pNPP) (267 μ l).

Finally, we want to end up with a kit that contains only 2 tubes.

- Tube #1: Tris reaction buffer (69 μ l), β -mercaptoethanol (4.4 μ l), and PP-1 (67 μ l).
- Tube #2: para-nitrophenyl phosphate (pNPP) (267 μ l).
- In addition we have established that:
 - pNPP substrate in tube #3 is stable frozen for 1 year.
 - A 3 ml plastic syringe is suitable to draw up the water.
 - A 0.45 mm millipore filter is optimal for sample preparation.
 - 4 ml test tubes with a non drip cap work best for sample manipulation.
 - Hot hands heat packs work efficiently. In the laboratory the hot packs tend to range from 42°C to 50°C.

Sensitivity and Time

The current sensitivity of the field assay stands at 1.5 μ g/L. The optimal reaction time is 30 minutes.

Future Work

- Stability of reaction buffer at 4°C.
- Stability of β -mercaptoethanol at 4°C.
- Sensitivity of PP-1 at various temperatures between 45-65°C.
- Stability of pNPP at 4°C.
- Stability of reaction buffer + β -mercaptoethanol at 4°C.
- 2 tube kit.
- Shorten the reaction time of testing.
- Sensitivity of detection below 1.0 μ g/L.

Standard Curve

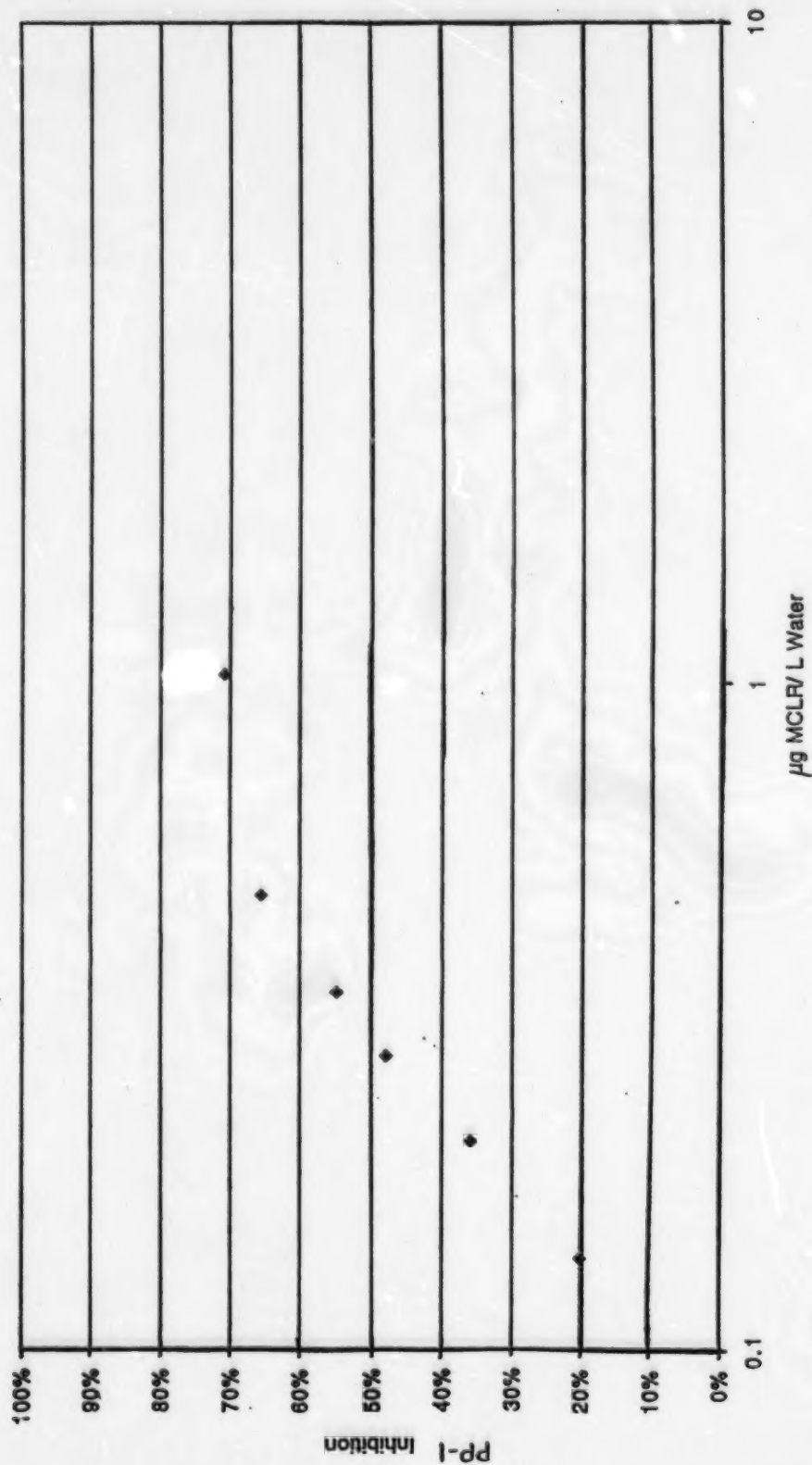


Figure 1 : Analysis of Mic-LR Between 0.15 µg/L - 1.0 µg/L.

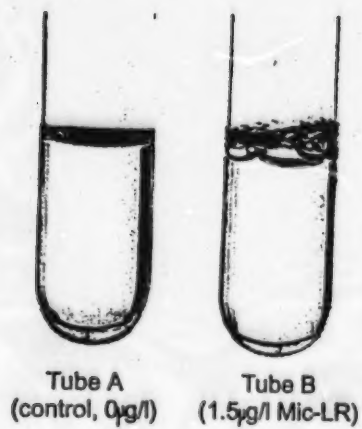


Figure 2: Field kit reaction after one hour incubation in heat pack.



Figure 3: Field kit reaction after one hour incubation in heat pack then left for several hours at room temperature.